



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/74, 15/76, 15/77	A1	(11) International Publication Number: WO 91/13157 (43) International Publication Date: 5 September 1991 (05.09.91)
--	-----------	--

(21) International Application Number: PCT/AU91/00064

(22) International Filing Date: 25 February 1991 (25.02.91)

(30) Priority data:
PJ 8815 26 February 1990 (26.02.90) AU

(71) Applicant (for all designated States except US): COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU).

(72) Inventors; and

(75) Inventors/Applicants (for US only): RADFORD, Anthony, John [AU/AU]; 2 Pakington Street, Kew, VIC 3101 (AU). HODGSON, Adrian, L., M. [AU/AU]; 22 Albert Street, East Malvern, VIC 3145 (AU).

(74) Agents: STEARNE, Peter, Andrew et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

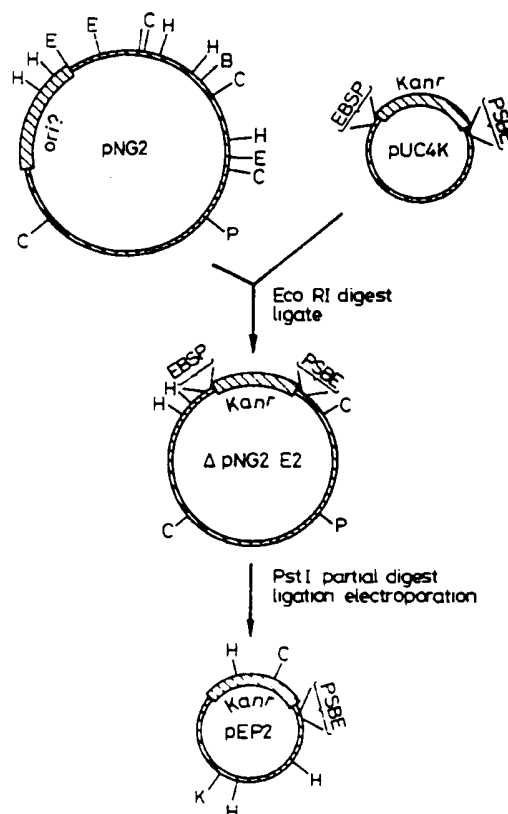
Published

With international search report.

(54) Title: SHUTTLE PLASMID FOR ESCHERICHIA COLI AND MYCOBACTERIA

(57) Abstract

DNA shuttle vectors which are capable of replication in Mycobacteria, *E. coli* and other bacterial hosts are described. The shuttle vectors comprise a single origin of replication which confers the ability of replication in a number of bacterial species. Also disclosed are shuttle vectors encoding desired polypeptides, such as antigens of disease causing bacteria, viruses and parasites.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

- 1 -

5

10

SHUTTLE PLASMID FOR ESCHERICHIA COLI AND MYCOBACTERIA

15

This invention relates to DNA vectors, and is particularly concerned with DNA shuttle vectors which are capable of replication in mycobacteria and Eschrichia coli cells. The invention also relates to bacterial hosts containing such DNA vectors, and further relates to vaccines containing such bacteria.

Mycobacterium bovis BCG (hereinafter BCG) has been used for many years as a vaccine against tuberculosis (TB). The vaccination programme has been extremely effective in controlling human TB firstly because BCG stimulates long term cell-mediated immunity and secondly because it has had an outstanding safety record. These characteristics make BCG a good candidate to form the basis of a live delivery system for recombinant vaccines.

A number of problems are associated with DNA manipulations involving mycobacteria. These include very few (one) plasmid vectors, poor growth rates of mycobacteria and low transformation rates when compared to bacterium such as E. coli. Given these problems, it is desirable to produce a shuttle vector which is capable of replication in a standard bacterial work horse such as E. coli for day to day genetic manipulations, and is

- 2 -

further capable of replication in Mycobacterium at or near the final stage of genetic manipulation. In this way, genes can be inserted into mycobacteria and time delays associated with Mycobacterium growth and transformation can be largely avoided. Gicquel-Sanzey et al. (Acta Lepologica 1989, 7, Suppl (1): 208-211) have described a mycobacteria-E. coli plasmid shuttle vector known as pAL8 which comprises two origins of replication, the first for mycobacteria and the second for E. coli. Multiple origins of replication have been necessary due to the evolutionary distance between mycobacteria and E. coli such that a mycobacterial plasmid having a mycobacterial origin is not capable of growth in E. coli and vice versa. Such vectors suffer from the problem that they are a considerable size due to the inclusion of two origins of replication, this decreasing cloning efficiency, the size of desired DNA fragments which may be inserted into such plasmids, and also increasing the number of unique restriction sites which may be introduced into the plasmids.

The present applicant has overcome problems associated with prior art shuttle vectors by providing a DNA shuttle vector which carries a single origin of replication which confers the ability of the vector to replicate in mycobacteria and E. coli cells. In a particularly preferred aspect of this invention as will be described hereinafter, the replication region corresponds to that of the corynebacterial plasmid pNG2 or fragments thereof.

In accordance with a first aspect of this invention, there is provided a DNA shuttle vector carrying a replication region comprising a single origin of replication, said origin of replication allowing replication of said vector in Mycobacteria and E. coli cells; and a selectable marker. The shuttle vector may additionally comprise a nucleotide sequence containing one or more restriction sites for the insertion of a

- 3 -

desired nucleotide sequence.

Preferably, the replication region corresponds to the replication region of the corynebacteria plasmid pNG2 or a fragment thereof which permits replication of said
5 vector in mycobacteria and E. coli.

Surprisingly, the replication region of plasmid pNG2 confers the ability of replication in various bacterial species, apart from Corynebacteria.

The DNA shuttle vector may further comprise a
10 promoter with one or more restriction endonuclease sites downstream of said promoter, such that when a nucleotide sequence is inserted into one or more of these sites, the promoter allows DNA transcription to proceed. The DNA shuttle vector may contain multiple promoters and
15 downstream restriction endonuclease sites.

The term "shuttle vector" as used herein includes plasmid DNA which may be double-stranded linear or double-stranded circular. The shuttle vector may be introduced into a bacterial cell by any number of
20 techniques well known in the art, such as conjugation, mobilisation, transformation, transfection, transduction or electroporation.

The term "selectable marker" as used herein refers to any selectable characteristic provided by or encoded
25 for, by a nucleotide sequence. Suitable detectable markers include resistance to antibiotics or enzymes or immunologically detectable proteins or chemicals capable of causing a detectable reaction when provided with a suitable substrate. Examples include resistance to
30 ampicillin, streptomycin, penicillin, hygromycin, kanamycin, and the like, β -galactosidase, urease, alkaline phosphatase and the like.

The term "promoter" is used in its broadest sense and refers to any nucleotide sequence which binds to RNA
35 polymerase and which directs the transcription of nucleotide sequences downstream (3' "or operably linked") to the promoter. Suitable promoters include prokaryotic

- 4 -

promoters such as the P1 promoter of bacteriophage
lambda, trp promoter, lac promoter, kanamycin resistance
promoters of transposon Tn903 and transposon Tn5,
mycobacterial promoters such as that of the promoter of
5 the common mycobacterial 65Kd antigen, ribosomal RNA
promoter of mycobacteria, promoters of M.bovis antigens
MPB70, MPB59 and MPB64 and the like, hybrids between
eukaryotic and prokaryotic promoters, and eukaryotic
promoters such as the metallothionine promoter, growth
10 hormone promoter, and the like.

Restriction endonuclease sites provided on the
vector may correspond to the cleavage of one or more
known restriction endonucleases, such as EcoR1, BamH1,
Pst1, Cla1, Kpn1, HindIII, HincI and HincII, and the
15 like. Restriction endonuclease sites may be provided in
the form of one or more polylinkers which contain a
number of closely grouped restriction endonuclease sites.

Nucleotide sequences of interest may be inserted
into the endonuclease cleavage sites provided on the
20 vector by ligation of DNA fragments having complementary
"sticky ends" to allow annealing thereof, or by ligation
of nucleotide sequence having "flush" ends (ends having
no unpaired nucleotides) by methods well known in the
art, and described for example, in Sambrook et al.
25 (Molecular Cloning: A Laboratory Manual, Cold Spring
Harbor Laboratory, 2nd Edition, 1989). A nucleotide
sequence for insertion into the vector of this invention
may include a promoter to direct transcription of
downstream (3') sequences. Promoters may be the natural
30 promoter of the gene to be transcribed or may be a
different promoter. As previously mentioned, the shuttle
vector of this invention may itself contain one or more
promoters upstream (5') from nucleotide sequences
encoding one or more restriction endonuclease cleavage
35 sites. In such an embodiment, a desired nucleotide
sequence lacking a promoter may be inserted into the
vector with transcription of the desired nucleotide

- 5 -

sequence being driven by the promoter resident on the vector.

Nucleotide sequences for insertion into the vector of this invention may, for example, encode one or more
5 antigens from disease causing bacteria, viruses or parasites, such as Taenia ovis, rotavirus, Babesia bovis, Mycobacterium bovis, Mycobacterium tuberculosis, Mycobacterium leprae, Mybacterium paratuberculosis, Bacteriodes nodosus or Bordetella. Desired nucleotide
10 sequences may also encode hormones such as LHRH, growth hormone or epitopes or analogues thereof; or DNA sequences capable of recombination with nucleotide sequences within a bacterial host cell; or nucleotide sequences capable of mutagenising DNA sequences within a
15 host cell. Where the shuttle vector of this invention functions as an expression vector, nucleotide sequences encoding desired products may include a signal or leader sequence to allow insertion into membranes of a suitable host cell or secretion from a host cell. Absence of a
20 secretory leader will cause the accumulation of antigen or other protein product within the cytoplasm of a bacterial host cell, where it may be recovered by well known methods.

In accordance with a specific embodiment of this
25 invention there is provided a DNA shuttle vector pEP2, said vector having a size of about 3.1 kb, as determined by agarose gel electrophoresis, a replication region of about 1.85 kb comprising a single origin of replication derived from the Corynebacterium replicon pNG2, an
30 antibiotic resistance gene to kanamycin, and a nucleotide sequence containing a number of restriction endonuclease cleavage sites for the insertion of a DNA sequence of interest. The 4.5 kbr plasmid pEP3 contains the same sequences of replication as pEP2 plus a marker encoding
35 hygromycin resistance effective in both E.coli and mycobacteria.

Shuttle vectors of this invention are capable of

- 6 -

replication in species of both gram negative and gram positive hosts, such as Mycobacterium, E. coli, Corynebacterium, and Actinomycetes, as defined in Bergey's Manual of Determinative Bacteriology, 8th Edition, pp. 599-861. The vectors of this invention do not appear to replicate in Bacillus species. Any bacterial strain may be readily tested according to methods well known in the art, to ascertain whether or not the shuttle vector of this invention is capable of replication therein. Advantageously, the shuttle vectors of this invention are capable of replication to high copy numbers in bacterial strains such as in the attenuated strain of Mycobacterium bovis BCG, which as previously stated has been extensively used in vaccination programmes throughout the world, and is a potent adjuvant, which stimulates long-term cell mediated immunity.

Shuttle vectors incorporating the origin of replication of pNG2 are particularly efficient in organisms of the genus Corynebacterium.

In accordance with a further aspect of this invention, there is provided a bacterial host which contains a shuttle vector as herein defined. The shuttle vector may be present as a single copy, or more preferably as multiple copies thereof within the bacterial host. Preferably, but in no way limiting the invention, the bacterial host is a Mycobacteria, Corynebacteria or E. coli strain, such as C. pseudotuberculosis, M. smegmatis, and M. bovis BCG. The vector may be used to deliver antigen or other protein genes into the bacterial host for expression thereof, as an excreted product from the host cell as previously described. Proteins may be expressed while residing on the plasmid vector or after recombination or insertion into the chromosome. Alternatively, expressed products may be inserted into or associated with the host cell membrane or cell wall or resident within the host cell

- 7 -

itself. Bacterial hosts expressing desired antigens may be provided as vaccines, for example, as M. bovis BCG, expressing a desired antigen. On immunisation, an immune response would be mounted to the host, such as M. bovis
5 BCG as well as the desired antigen of the disease causing bacterium, virus or parasite.

In a further aspect of this invention there is provided a polypeptide when expressed by a bacterial host cell containing a shuttle vector as herein defined.

10 This invention contemplates deletions or insertions of nucleotide sequences to or from the replication region of pNG2 as long as such modifications do not prevent the ability of such sequences to confer replication in Mycobacterium and E. coli. Techniques for insertion or
15 deletion of nucleotide sequences are well known in the art. Mutants could be readily tested for the ability to confer replication in gram negative and gram positive host cells, such as E. coli and corynebacteria.

This invention also extends to replication region of
20 pNG2 itself or fragments thereof, which, on insertion into suitable vector are capable of permitting replication of said vector mycobacteria and E. coli.

A culture of Eschrichia coli containing plasmid pEP2 was deposited under the terms and conditions of the
25 Budapest Treaty at the Australian Government Analytical Laboratory (AGAL), Pymble, New South Wales, Australia on 23rd February, 1990 and accorded Accession No. N90/007080.

This invention will now be illustrated with
30 reference to the following non-limiting Figures and Examples.

FIGURES

Figure 1 shows the construction of plasmid pEP2. Plasmid pNG2 (14.5) was digested with EcoRI and the
35 largest fragment ligated to the Kan^r cartridge of pUC4K. Following electroporation into E.coli the resulting plasmid DNA was extracted and partially digested with

- 8 -

PstI, and then religated and again electroporated with E.coli. One of the resulting Kan^r colonies contained plasmid pEP2. Restriction sites: B(BamHI), E(EcoRI), H(HindIII), Hc(HincII), P(PstI), S(SalI). Kan refers to
5 kanamycin resistance gene from pUC4K.

Figure 2A is a circular map of the Mycobacterium-E.coli shuttle vector pEP2. The restriction map is given only for relevant cleavage sites: B (BamHI), E. (EcoRI), H (HindIII), Hc (HincII), K (KpnI), p (PstI), S (SalI).
10 Kanamycin resistance gene \\\ from pUC4K.

B Sequencing strategy. Arrows indicate the extent and direction of sequence generated from restriction fragments cloned in M13 vectors. X denotes sequence derived using oligonucleotide primers.

15 Figure 3 is a complete nucleotide sequence of the replication region of pEP2. Relevant restriction sites are marked for comparison with Figure 1 (E (EcoRI), H (HindIII), Hc (HincII), K (KpnI). IR and DR, inverted and direct repeat sequences respectively; RBS, putative
20 ribosome binding site; -----> <----- dyad symmetry associated with putative rho-dependent transcriptional terminator, T. ORFA, major open reading frame.

Figure 4 shows an agrose gel (right hand plate) and a Southern blot (left hand plate) of that gel probed with
25 plasmid pEP2 extracted from E.coli. Tracks contain (A) Undigested pEP2 DNA from E.coli 500ng, (B) Pst 1 digested pEP2 DNA from E.coli 200ng, (C) PstI digested whole DNA extract of M. bovis BCG pEP2, 2.5ug, (D) PstI digested M. bovis BCG DNA, 2.5 ug (E) Undigested DNA extract of M. bovis BCG pEP2, 2.5ug, (F) Undigested DNA extract of M. bovis BCG, 2.5ug, (G) HindIII digested lambda DNA
30 markers.

EXAMPLE 1

Recombinant DNA Procedures:

35 Unless otherwise specified herein, manipulation of recombinant molecules and the preparation of solutions are by standard known techniques. Such techniques are

- 9 -

described in Sambrook et al. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor [1989], pp. 1-500.

Bacterial Strains and Plasmids:

- 5 Mycobacterium bovis BCG variant CSL was obtained as lyophilised human vaccine from the Commonwealth Serum Laboratories (CSL) Australia. M. smegmatis and M. pheli were obtained from the Fairfield Hospital, Melbourne, Australia and Escherichia JM109 was obtained from Promega
- 10 (Madison, Wisconsin, U.S.A.). Plasmids pNG2 (Serwold-Davis et al. 1987, Proc. Natl. Acad. Sci. USA 84: 4964-4968) and pPB3 was obtained from Dr. Philip Bird (Monash University Faculty of Medicine, Alfred Hospital, Melbourne). Plasmid pAL8 (Gicquel-Sanzey et al. 1989
- 15 Acta Leprol. 7: 207-211) was obtained from Dr. Brigitte Gicquel-Sanzey (Pasteur Institute, Paris) and plasmid pUGC4K was purchased from Pharmacia LKB (Uppsala, Sweden).

Media:

- 20 E. coli strains were grown Luria broth, (LB:10 grams tryptone, 5 grams yeast extract, 10 grams NaCl per litre). Coryneform bacteria were cultured in LB media (Oxoid) and mycobacterium species were grown in Dubos or 7H11 media (Oxoid, Australia).

25 **Electroporation of Bacteria:**

- Mycobacterium species and Corynebacterium pseudo-
tuberculosis were electroporated according to Lugosi et al. (Tubercule 70: 159-170 [1989]) using a Gene Pulser commercially available from Bio-Rad Laboratories Inc.
- 30 Transformants were selected on 7H11 or nutrient media containing 100 µg kanamycin per ml or 200µg hygromycin B per ml.

DNA Isolation and Hybridization Analysis:

- DNA was extracted from Mycobacterium using a
- 35 modification of a method used to isolate DNA from yeast (Mann and Jeffrey, Anal. Biochem. 1981, 178: 82-87).
- Mycobacterial cells were harvested from 400 mls of Dubos

- 10 -

broth by centrifugation, resuspended in 5 ml of TE buffer and heat-killed by treatment at 70°C for 1 hour. Glass beads (3-6 mm) were added and the mix vortexed vigorously for a minimum of 30 seconds to disperse the bacteria.

- 5 Bacterial suspensions were then transferred to liquid nitrogen in a mortar pre-cooled in a bath of liquid nitrogen and crushed into a fine powder using a pre-cooled pestle. The crushing of cells was performed in a biohazard hood. Frozen crushed cells were added in small
- 10 portions (spatula loads) to 15 ml of lysis buffer (6.6 mM Tris, 30 mM EDTA, 1.2% w/v sodium lauroylsarkosinate). Five mg of protease K was added and the mixture incubated for 90 minutes at 37°C. The mix was then extracted with an equal volume of phenol-chloroform and the aqueous
- 15 phase precipitated with isopropanol at 4°C for 10 minutes. The pellet was dissolved in 1.0 ml of TE buffer and extracted with phenol-chloroform and then water-saturated ether prior to ethanol precipitation and resuspension in TE.

- 20 Genomic DNA was isolated from C. pseudotuberculosis as previously described (Hodgson et al., 1990).

- pNG2RI DNA was digested with various restriction enzymes and the fragments Southern blotted (Reid et al.) to Hybond N (Amersham) nylon filters. Filters were
- 25 hybridised overnight at 37°C with pEP2 labelled with 32P using random hexamer primers, washed at increasing stringency as necessary (up to 65°C) and exposed to X-ray film (Fuji RX). Restriction digested total genomic DNA isolated from M. bovis BCG and that transformed with pEP2
- 30 was Southern blotted to nylon and probed with labelled pEP2 as described above. Other transformed Mycobacterium species were analysed with the pEP2 probe using DNA dot blot hybridisation. C. pseudotuberculosis transformants were analysed in the same fashion.

- 35 Total cell DNA was used to isolate and transform E.coli to kanamycin or hygromycin resistance.

- 11 -

DNA Sequence Analysis:

Nucleotide sequence analysis was determined by the dideoxy chain termination method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA 74: 5463-5467) using modified
5 T7 DNA polymerase (Pharmacia LKB, Uppsula, Sweden). The complete nucleotide sequence of pEP2 was derived on both strands using universal or oligonucleotide primers (Figure 1B). The latter was synthesized using a Gene Assembler plus DNA Synthesizer (Pharmacia LKB, Supra).
10 DNA and amino acid sequence data were collated and analysed using the DNASIS and PROSIS software packages (Pharmacia LKB).

EXAMPLE 2**Shuttle Vector Construction and Analysis Thereof:**

15 The 1.3 kb EcoR1 fragment from pUC4K carrying the kanamycin resistance gene was ligated to pNG2 DNA digested with EcoR1. The ligation mix was electroporated into E.coli JM109 and transformant selected on LB plates supplemented with 50µg kanamycin per ml. All
20 transformants contained plasmids with the 9.5 kb pNG2 EcoR1 fragment. Plasmid DNA (2 µg) from one of these clones (pNG2RI) was partially digested with PstI and blunted using T4DNA polymerase. DNA fragments smaller than 10 kb were purified from a 1% agarose gel and used
25 to transform E. coli JM109 to kanamycin resistance. A restriction map of a resulting plasmid (pEP2) isolated from a transformant was derived using standard procedures (Sambrook et al., Supra) and is shown in Figure 1A.

Plasmid pEP2 has a molecular weight of 3.1 kb as
30 determined by agarose gel electrophoresis. This plasmid retains one of the pUC4K polylinkers and hence has unique PstI, SalI, BamHI and EcoRI sites. Hybridization analysis shows that plasmids pEP2 and pNG2RI (pNG2 after digestion with EcoRI) both contain an 800 bp HindIII
35 fragment. Southern blotting of digests of sub-clones of pNG2 with the pEP2 plasmid showed that the region of pNG2 marked in Figure 1 was that incorporated in the pEP2

- 12 -

plasmid, and thus the area containing the origin of replication.

To determine whether hygromycin resistance could be used as a selectable marker in Mycobacteria species and
5 C. pseudotuberculosis a hygromycin phosphotransferase gene (hph) was cloned into the Mycobacterium shuttle vector pEP.

To clone the hygromycin phosphotransferase gene (hph) from pPB3 into pEP2, pUC4K DNA was linearised with
10 XhoI and then blunted using the Klenow fragment of DNA polymerase I. pPB3 DNA was digested for 30s on ice using 5 units each of AluI, HaeIII and RsaI. Fragments 2.0kb in size were gel purified (Geneclean, Bresatech) and ligated overnight with the linear, blunted pUC4K DNA. The
15 ligation mix was electroporated into JM109 and recombinants were selected on LB plates supplemented with 150ug hygromycin B (Sigma) per ml. A 1.7kb SalI-ClaI insert from a pUC4K chimera was ligated into the SalI-ClaI site within the kanamycin resistance gene of pEP2.
20 JM109 was transformed to hygromycin resistance with the ligation mix as described above and a restriction map of a hygromycin resistance plasmid (pEP3) was derived. The plasmid pEP3 has a unique SalI site and is approximately 4.5 kb in size (Fig. 2).

25 The nucleotide sequence of pEP2 excluding the kanamycin resistant gene is presented in Figure 3. Examination of the DNA sequence revealed a single open reading frame (ORF). A number of potential translational start codons can be identified within this region but
30 only one is preceded by a putative ribosome binding site (Figure 2). Although the sequence upstream of this ORF (ORFA) does not possess an E. coli consensus promoter, a putative rho-dependent transcriptional terminator was found downstream of the stop codon (Figure 2). This
35 putative terminator has a 90% match to the TAATCAATAT consensus sequence (Ryder et al., Initiation of DNA Replication [1981], Academic Press, New York) and as has

- 13 -

been described for other rho-dependent terminators (Rosenberg et al., Nature 272, 414-428 [1978]), and is preceded by a region of dyad symmetry (Figure 2). ORFA is therefore capable of coding for a 28kDa protein, a size consistent with that reported for other Rep proteins.

In addition to the predicted size of the ORFA protein, further evidence to suggest that we have identified the legitimate translated region arises from an examination of the derived amino acid sequence. Firstly, the codon bias for the putative ORFA product (Phe, TTC; Asp, GAC; Arg, CGC; Ile, ATC; Val, GTC; Ala, Gcc; Thr, ACC) is the same as that for other Corynebacterium proteins. Secondly, the predicted protein encoded by ORFA is highly basic in nature (19% basic residues) which is a characteristic of Rep proteins (and other DNA binding proteins) thought to be important in the role they play in replication and incompatibility.

Database searches were performed using both the complete 1.85kb nucleotide sequence and the predicted amino acid sequence of ORFA, however no significant homologies were found. In addition, more detailed analyses revealed no similarities between the pEP2 Rep region and either of the potentially related plasmids from Corynebacterium (pBL1, Martin et al., Biotechnol. 5: 137-146 [1987]) and Mycobacterium (pAL5000, Rauzier et al., Gene II: 315-321 [1988]).

Plasmid replication regions invariably have an origin of replication. Most commonly, origins are located in non-coding regions, possess clusters of direct and inverted repeats and may be preceded by A+T rich sequences (Kamio et al., J. Bacteriol. 258: 307-312 [1984], Scott et al., Microbiol. Rev. 48: 1-23 [1984], Rosen et al., Mol. Gen. Genet. 179: 527-537 [1980], Rauzier et al., Gene 71: 315-321 [1988]). The region upstream of ORFA contains no ORFs and possesses a number of direct and inverted repeat DNA sequences (Figure 2).

- 14 -

In addition, the region between nt 86 and 171 is 63.5% A+T compared with an average of 45% for the entire Rep region (Figure 2). We therefore believe this to be the pEP2 origin of replication.

5 The replication region of pEP2 is capable of encoding a single 28kDa protein and possesses a single origin allowing plasmid replication in both Gram-positive and negative bacteria. pEP2 is therefore a unique shuttle vector and should be a useful tool, for example,
10 in the genetic analysis of Mycobacterium and in the development of M. bovis BCG as a live recombinant vaccine.

Electroporation of Mycobacteria with pEP2 and pEP3:

Electroporation of Mycobacterium species with pEP2
15 DNA resulted in transformation to kanamycin resistance respectively (Table 1). To confirm that the plasmids were present in the mycobacteria, total cellular DNA preparations were made from the kanamycin resistant transformants. Figure 4 shows the results of agarose gel
20 and Southern blot analysis of total cell DNA extracted from BCG CSL strains that had been transformed with pEP2. The pEP2 plasmid is clearly present in the transformed strains. Furthermore, the relative intensity of the gel bands suggests that the pEP plasmids replicate to high
25 copy number in these bacteria. In addition, when total genomic DNA isolated from the drug resistant transformants was used to electroporate E. coli, approximately 1.0×10^5 kanamycin resistant clones were obtained per ug DNA. Taken together these data suggest
30 that the pEP2 replicon promotes stable plasmid replication to high copy number in these bacteria.

Plasmid pEP3 was capable of transforming M. smegmatis and M. bovis BCG CSL to resistance to at least 200ug hygromycin per ml (Table 1). Total cellular DNA
35 isolated from pEP3 transformed M. smegmatis was capable of transforming E. coli to hygromycin resistance. This shows that the php gene encoding hygromycin resistance is

- 15 -

effective in both mycobacteria and E. coli and thereby constitutes a useful marker for these bacteria.

TABLE 1

ELECTROPORATION OF MYCOBACTERIA AND RELATED SPECIES

Plasmid electroporation efficiency: Kan^r CFU
per microgram of plasmid DNA

Bacterial Species	pEP2	pAL8	pEP3
<u>Mycobacterium phlei</u>	---	10 ³ 10 ²	10 ¹
<u>Mycobacterium smegmatis</u>	10 ² 10 ¹	10 ¹	10 ²
<u>Mycobacterium bovis</u>	10 ² 10 ²	10 ² 10 ³	N/D
BCG var CSL	10 ²	10 ⁴	10 ¹
<u>Corynebacterium ovis</u> (pseudotuberculosis)	10 ³ 10 ⁴	---	10 ²

N/D = Not done, --- = no transformation to resistant phenotype
Different figures for each electroporation represent the result
of separate experiments.

- 16 -

Electroporation of Other Bacterial Species with pEP2 and pEP3:

To determine the host range of the pEP replicon, pEP2 and 3 plasmid DNA was electroporated into Cornybacterium pseudotuberculosis. Transformation occurred in C. pseudo tuberculosis (Table 1). These results indicate that the pEP2 plasmids have a wide host range.

10

EXAMPLE 3

Generation and Expression of pEP2 Constructs:

A PCR derived DNA fragment carrying the promoter from the Mycobacterial 65kDa heat shock protein (0.6Kb) was cloned into the PstI-BamHI sites of pEP2, generating a plasmid referred to as pEP5. This promoter is known to function in E. coli as well as Mycobacteria and is therefore useful in this shuttle vector expression system.

A gene encoding for chloramphenicol-acetyl-transferase (CAT) was cloned into the BamHI site of pEP5. Expression of the CAT gene driven by the 65kDa promoter was detected using the Pharmacia (Registered trademark, Pharmacia, Pitcataway, N.J., U.S.A.) CAT detection kit. According to this assay the 64kDa promoter has a strength comparable with the induced lac promoter in both E. coli and C. pseudotuberculosis.

MPB70 is the major secreted protein of M. bovis and a component of PPD. This gene and its signal sequence was incorporated into the pEP2 expression system. A truncated form of the MPB70 gene was generated by PCR removing its promoter but leaving its ribosome binding site (RBS) intact. This was cloned into pEP2 as a PstI-BamHI fragment (0.5Kb). The 65kDa promoter was then cloned upstream of the MPB70 BS on a PstI-ScaI fragment.

After electroporation into E. coli and C. pseudotuberculosis, expression was tested for by western blots and Elisass. This construct did not express in E.

- 17 -

coli. It did however express in C. pseudotuberculosis with product detected in both the solicate and culture filtrate. This indicated that the MPB70 RBS was inactive in E. coli yet was recognised and functional in C.

5 pseudotuberculosis.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention
10 includes all such variations and modifications which fall within its spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification,
individually or collectively, and any and all
15 combinations of any two or more of said steps or features.

CLAIMS:

1. A DNA shuttle vector carrying a replication region comprising a single origin of replication, said origin of replication allowing replication of said vector in Mycobacteria and E.coli cells; and a selectable marker.

2. A shuttle vector according to claim 1 comprising a first nucleotide sequence operably linked to said origin of replication, and containing one or more restriction endonuclease cleavage sites for the insertion of a desired second nucleotide sequence.

3. A shuttle vector according to any one of claims 1 or 2 which comprises one or more promoters having operably linked thereto a nucleic acid sequence encoding a desired polypeptide.

4. A shuttle vector according to claim 1 wherein said selectable marker confers resistance to an antibiotic or enzyme, or encodes an immunologically detectable protein or chemical compound capable of causing a detectable reaction when provided with a suitable substrate.

5. A shuttle vector according to claim 4, wherein said selectable marker confers resistance to ampicillin, streptomycin, penicillin, hygromycin or kanamycin.

6. A shuttle vector according to claim 4, wherein said selectable marker is a gene encoding β -galactosidase, urease or alkaline phosphatase.

7. A shuttle vector according to claim 3, wherein said promoter is selected from the P1 promoter of bacteriophage lambda, trp promoter, lac promoter,

- 19 -

kanamycin resistance promoters of transposon Tn903 or transposon Tn5, mycobacteria promoter of the common mycobacterial 65Kd antigen, ribosomal RNA promoter of mycobacteria, promoter of M.bovis antigens MPB70, MPB59 and MPB64, metallothionine promoter, growth hormone promoter or hybrids between eukaryotic and prokaryotic promoters.

8. A shuttle vector according to claim 3, wherein said desired peptide encodes one or more antigens from disease causing bacteria, viruses or parasites.

9. A shuttle vector according to claim 8, wherein said one or more antigens are selected from antigens of Taenia ovis, rotavirus, Babesia bovis, Mycobacterium bovis, Mycobacterium tuberculosis, Mycobacterium leprae, Mybacterium paratuberculosis, Bacteriodes nodosus or Bordetella.

10. A shuttle vector according to claim 3, wherein said desired polypeptide is a hormone or an epitope thereof.

11. A shuttle vector according to claim 1, wherein said origin of replication comprises the replication region of the Corynebacterium plasmid pNG2 or a fragment thereof which permits replication of said vector in mycobacteria and E.coli.

12. A shuttle vector according to any one of claims 1 to 11, capable of replication in Actinomycetes.

13. A shuttle vector according to any one of claims 1 to 11, capable of replication in Corynebacteria.

14. A shuttle vector selected from pEP2 and pEP3.

- 20 -

15. A bacterial host cell containing a shuttle vector according to any one of claims 1 to 14.

16. A host cell according to claim 15 selected from Mycobacteria, E. coli, Corynebacteria, and Actinomycetes.

17. A host cell according to claim 15 selected from M. pheli, M. smegmatis, M. bovis BCG, E. coli JM109, C. pseudotuberculosis.

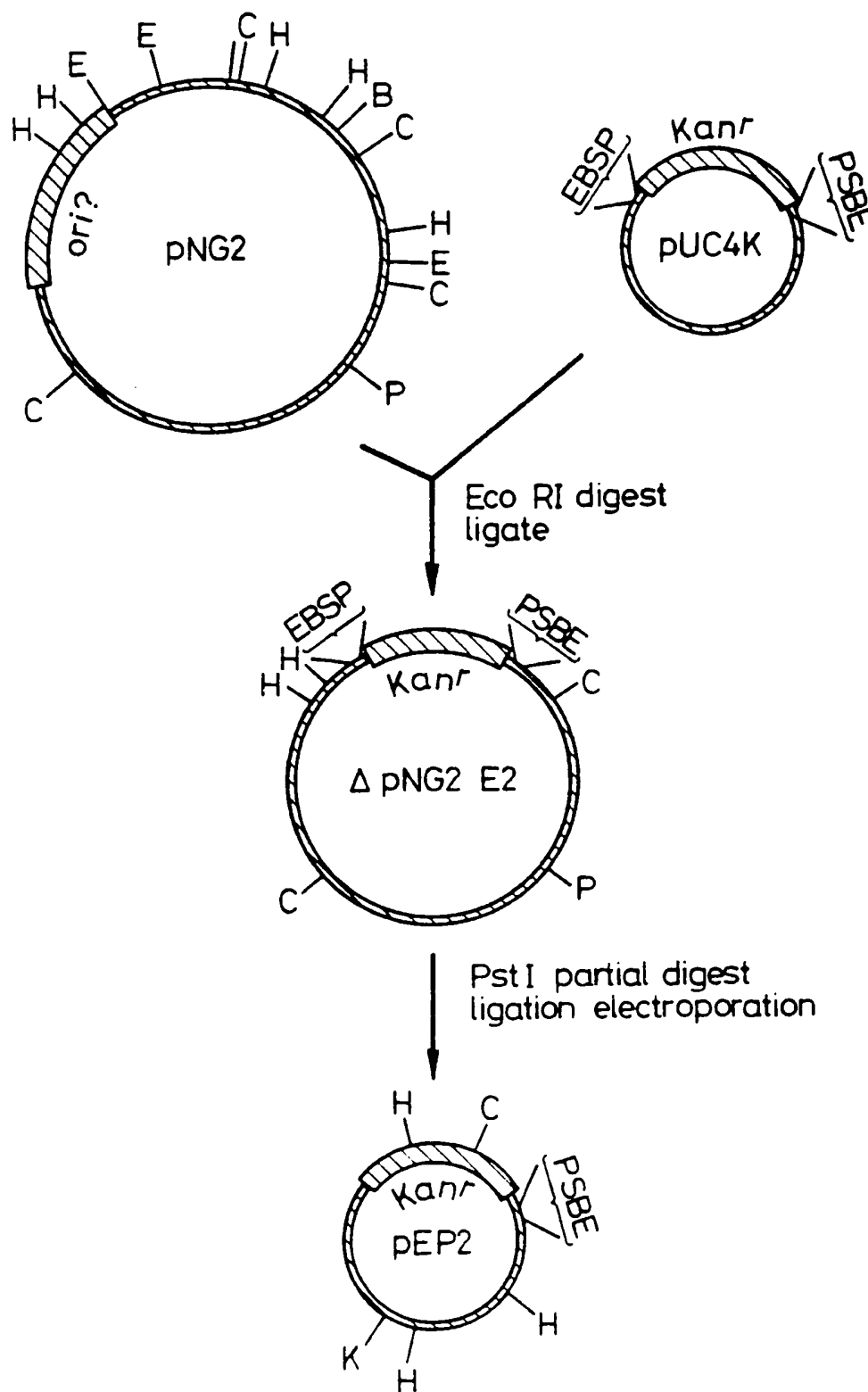
18. A DNA sequence encoding the origin of replication of Corynebacterium plasmid pNG2 or a fragment thereof which permits replication of a vector containing said DNA sequence in Mycobacteria and E. coli.

19. A DNA sequence according to claim 17 comprising an origin of replication operably linked to said origin of replication.

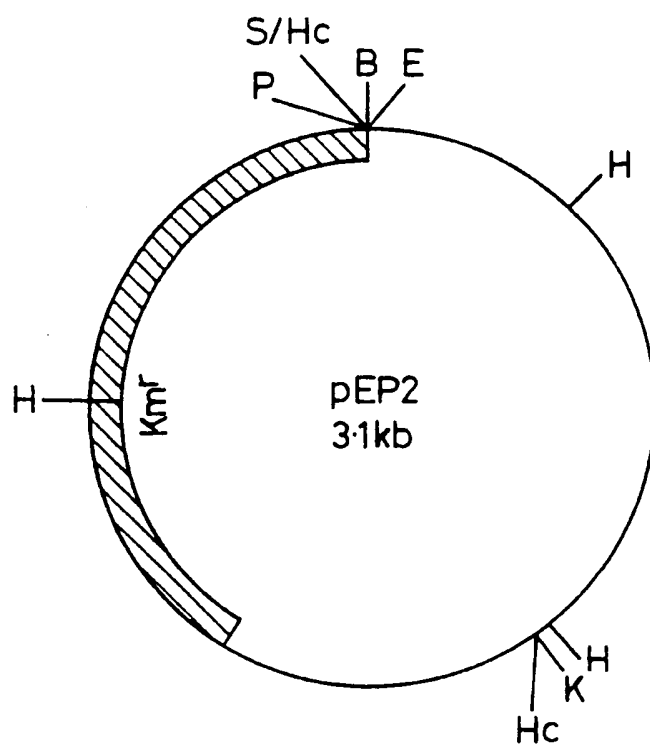
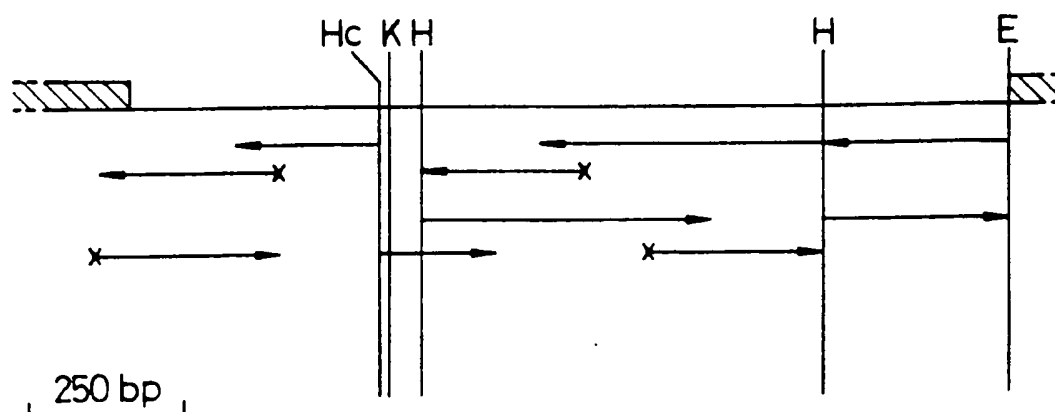
20. A DNA sequence according to claim 18 where the DNA sequence encodes a desired polypeptide is operably linked to said promoter.

21. A polypeptide expressed by a host cell wherein said polypeptide is encoded by a shuttle vector according to any one of claims 4 and 8 to 9, resident within said host cell.

1/9
Fig. 1.



2/9

*Fig.2A.**Fig.2B.*

3/9

1 ATG GTA AAT CTG CGC AGA CAG CCC TGT GCA GCT
IR1

97 TTT TTC GCG TGT CAT GGC TAG TAA ATA ACA CCG

193 GAC TTT CGC TTA TCA CCC AGC ACA CAC CTG GGA

289 TCG AGC GCG ACC GTG GTG GAC TGG ACA ACA CCC

385 CAG TCA CAC GAG ACT TTA AAA AGG CCT ATC GAC

481 CGA GTA CGC CAT GCT CAC CAC CAA GCA GTA CGC
DR3 DR3

577 CCG TAC GTC CGC GAC GTG GTG CGC TCA CTG ATT

673 TGG CTT ATT GAC CCT GTC TAC GCT GAC CGT AAC
IR2

769 CAT GAC CCG CAC TTT TCC CAC CGC TTT AGC CGC

865 ATG CGC CTT GGA GAC TTG ATA AAG CAG GTA AGG

Fig. 3a.

4/9

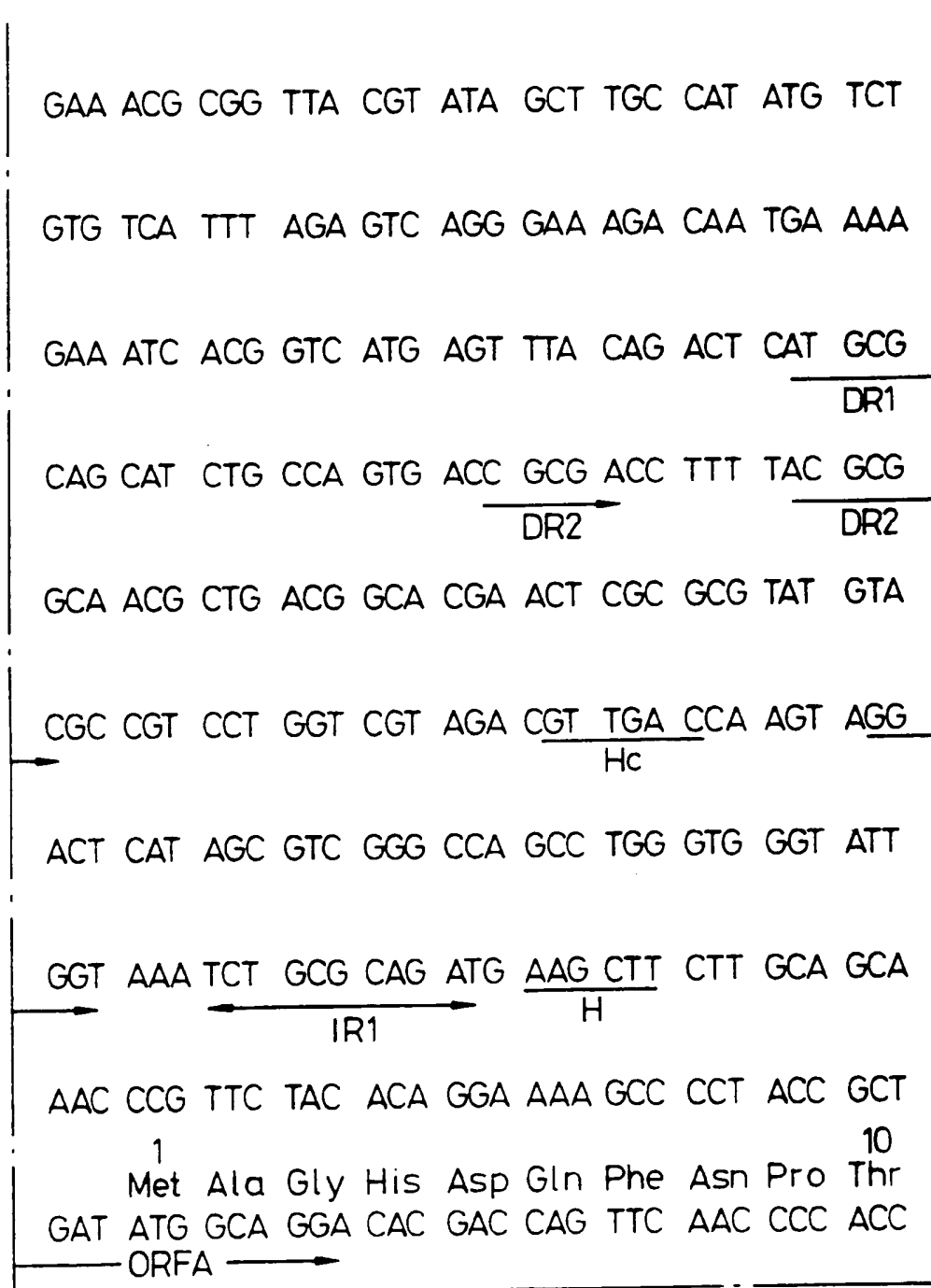


Fig.3b.

5/9

AGC CAT ACG TAA CCG CAG GTA AAA GGC ATA	96
ACG AAG AAA GCC ACC GGG CGG CAA CCC GAT	192
CAG AAT GCG CAC ACT AAA ACA CCT ACC GCG	288
→ DR1 →	
ATC ATC TAG GCC GCG ATG TAC TCC ACG GTT	384
→ DR2 →	
TCG CTT CGA GAC TGA TGC TTT AGG ACG GTG	480
TAC CGC AGC GGT GAC CCC GCA GAC TTA AAC	576
K → DR4 → DR4 →	
AAC CCA ACT AAC GGC AAA GCC CAG TTC ATA	672
ACC ACG CGT GTG CTG GGT GAG CTT TTA GAC	768
TAT CGT TGG TAT AGG CAG CAC AAC CGG GTG	864
Pro Arg Gln Gln Phe Ser Ser Gly Arg Glu	20
CCA CGC CAG CAA TTC AGC TCT GGC CGC GAA	960

Fig. 3c.

6/9

										30		
	Leu	Ile	Asn	Ala	Val	Lys	Thr	Arg	Arg	Glu	Glu	
961	CTT	ATC	AAC	GCG	GTC	AAG	ACC	CGC	CGT	GAA	GAA	
										60		
	Tyr	Asp	Pro	Glu	Leu	Ile	Asp	Gly	Val	Arg	Val	
1057	TAT	GAC	CCG	GAA	CTT	ATC	GAC	GGT	GTG	CGT	GTG	
										90		
	Ala	Thr	Val	Ala	Pro	Ala	Ser	Gln	Arg	Leu	Thr	
1153	GCC	ACC	GTT	GCG	CCA	GCA	AGC	CAA	CGC	CTG	ACA	
										120		
	Gly	Arg	Asp	Asn	Glu	Met	Pro	Pro	Met	Arg	Asp	
1249	GGC	CGC	GAC	AAC	GAG	ATG	CCA	CCC	ATG	CGC	GAC	
										150		
	Gly	Ser	Asn	Ala	Pro	Gly	Lys	Ala	Thr	Ser	Ser	
1345	GGC	TCT	AAC	GCA	CCA	GGT	AAA	GCC	ACC	AGC	AGC	
										190		
	Thr	Asp	Pro	Glu	Gly	Lys	Tyr	Ala	Gln	Ala	Gln	
1441	ACA	GAC	CCC	GAG	GGC	AAA	TAT	GCG	CAA	GCA	CAA	
										220		
	Ile	Ser	Gln	Met	Val	Asn	Asp	Gln	Tyr	Phe	Gln	
1537	ATT	AGC	CAA	ATG	GTG	AAC	GAT	CAG	TAT	TTC	CAG	
										250		
	Ala	Arg	His	Val	Ala	Glu	Leu	Lys	Lys	Ser	Gly	
1633	GCT	AGG	CAT	GTC	GCG	GAG	CTA	AAG	AAG	AGC	GGT	
1729	AGT	TAG	ATA	AAA	CCT	CAC	TTG	AAG	AAA	ACC	TTG	
1825	CTC	CGC	CAT	AAC	CTC	ACC	<u>GAA</u>	<u>TTC</u>				
							E					

Fig. 3d.

SUBSTITUTE SHEET

7/9

											40
Ala	Gln	Ala	Phe	Lys	Ala	Leu	Ala	Gln	Asp	Val	
GCC	CAA	GCA	TTC	AAA	GCA	CTC	GCC	CAG	GAC	GTA	
											70
Leu	Trp	Ile	Val	Gln	Gly	Thr	Ala	Ala	Arg	Asp	
CTC	TGG	ATT	GTC	CAA	GGA	ACC	GCA	GCA	CGC	GAC	
											100
Asp	Ala	Ala	Ile	Ile	Asp	Ala	Tyr	Glu	His	Ala	
GAC	GCA	GCA	ATC	ATC	GAC	GCC	TAT	GAG	CAC	GCC	
											130
Arg	Gln	Thr	Met	Ala	Arg	Arg	Val	Arg	Gly	Tyr	
CGC	CAA	ACC	ATG	GCA	AGG	CGC	GTG	CGC	GGG	TAT	
											160
Glu	Arg	Lys	Ala	Leu	Ala	Thr	Met	Gly	Arg	Arg	
GAG	CGG	AAA	GCC	TTG	GCC	ACG	ATG	GGA	CGC	AGA	
											170
											200
Arg	Ser	Lys	Leu	Glu	Lys	Thr	His	Arg	Lys	Lys	
AGG	TCG	<u>AAG CTT</u>	GAA	AAG	ACG	CAC	CGT	AAG	AAA		
											230
Thr	Gly	Thr	Val	Pro	Thr	Trp	Ala	Glu	Ile	Gly	
ACA	GGG	ACA	GTT	CCC	ACG	TGG	GCT	GAA	ATA	GGG	
											260
Asp	Tyr	Pro	Asp	Val	***	260					
GAC	TAT	CCG	GAC	GTT	TAA	GGG	GTC	TCA	TAC	CGT	
											290
											320
											350
											380
											410
											440
											470
											500
											530
											560
											590
											620
											650
											680
											710
											740
											770
											800
											830
											860
											890
											920
											950
											980
											1010
											1040
											1070
											1100
											1130
											1160
											1190
											1220
											1250
											1280
											1310
											1340
											1370
											1400
											1430
											1460
											1490
											1520
											1550
											1580
											1610
											1640
											1670
											1700
											1730
											1760
											1790
											1820
											1850
											1880
											1910
											1940
											1970
											2000
											2030
											2060
											2090
											2120
											2150
											2180
											2210
											2240
											2270
											2300
											2330
											2360
											2390
											2420
											2450
											2480
											2510
											2540
											2570
											2600
											2630
											2660
											2690
											2720
											2750
											2780
											2810
											2840
											2870
											2900
											2930
											2960
											2990
											3020
											3050
											3080
											3110
											3140
											3170
											3200
											3230
											3260
											3290
											3320
											3350
											3380
											3410
											3440
											3470
											3500
											3530
											3560
											3590
											3620
											3650
											3680
											3710
											3740
											3770
											3800
											3830
											3860
											3890
											3920
											3950
											3980
											4010
											4040
											4070
											4100
											4130
											4160
											4190
											4220
											4250
											4280
											4310
											4340
											4370
											4400
											4430
											4460
											4490
											4520
											4550
											4580
											4610
											4640
											4670
											4700
											4730
											4760
											4790
											4820
											4850
											4880
											4910
											4940
											4970
											5000
											5030
											5060
											5090
											5120
											5150
											5180
											5210
											5240
											5270
											5300
											5330
											5360
											5390
											5420
											5450
											5480
											5510
											5540
											5570
											5600
											5630
											5660
											5690
											5720
											5750
											5780
											5810
											5840
											5870
											5900
											5930
											5960
											5990
											6020
											6050
											6080
											6110
											6140
											6170
											6200
											6230
											6260
											6290
											6320
											6350
											6380
											6410
											6440
											6470
											6500
											6530
											6560
											6590
											6620
											6650
											6680
											6710
											6740
											6770
											6800
											6830
											6860
											6890
											6920
											6950
											6980
											7010
											7040
											7070
											7100
											7130
											7160
											7190
											7220
											7250
											7280
											7310
											7340
											7370
											7400
											7430
											7460
											7490
											7520
											7550
											7580
											7610
											7640
											7670
											7700
											7730
											7760
											7790
											7820
											7850
											7880
											7910
											7940
											7970
											8000
											8030
											8060
											8090
											8120
											8150
											8180
											8210
											8240
											8270
											8300
											8330
											8360
											8390
											8420
											8450
											8480
											8510
											8540
											8570
											8600
											8630
											8660
											8690
											8720
											8750
											8780
											8810
											8840
											8870
											8900
											8930
											8960
											8990
											9020
											9050
											9080
											9110
											9140
											9170
											9200
											9230
											9260
											9290
											9320
											9350
											9380
											9410
											9440
											9470
											9500
											9530
											9560
											9590
											9620
											9650
											9680
											9710
											9740
											9770
											9800
											9830
											9860
											9890
											9920
											9950
											9980
											10010
											10040
											10070
											10100
											10130
											10160
											10190
											10220
											10250
											10280
											10310
											10340
											10370
											10400
											10430
											10460
											10490
											10520
											10550
											10580
											10610
											10640
											10670
											10700
											10730
											10760
											10790
											10820
											10850
											10880
											10910
											10940
											10970
											11000
											11030
											11060
											11090
											11120
											11150
											11180
											11210
											11240
											11270
											11300
											11330
											11360
											11390
											11420
											11450
											11480
											11510
											11540
											11570
											11600
											11630
											11660
											11690
											11720
											11750
											11780
											11810
											11840
											11870
											11900
											11930
											11960
											11990
											12020
											12050
											12080
											12110
											12140
											12170
											12200
											12230
											12260
											12290
											12320
											12350
											12380
											12410
											12440
											12470
											12500
											12530
											12560
											12590
											12620
											12650
											12680
											12710
											12740
											12770
											12800
											12830
											12860
											12890
											12920
											12950
											12980
											13010
											13040
											13070
											13100
											13130
											13160
											13190
											13220
											13250
											13280
											13310
											13340
											13370
											13400
											13430
											13460
											13490
											13520
											13550
											13580
											13610
											13640
											13670
											13700
											13730
											13760
											13790
											13820
											13850
											13880
											13910
											13940
											13970
											14000
											14030
											14060
											14090
											14120
											14150
											14180
											14210
											14240
											14270
											14300
											14330
											14360
											14390
											14420
											14450
											14480
											14510
											14540
											14570
											14600
											14630
											14660
											14690
											14720
											14750
											14780
											14810
											14840
											14870
											14900
											14930
											14960
											14990
											15020
											15050
											15080
											15110
											15140
											15170
											15200
											15230
											15260
											15290
											15320
											15350
											15380
											15410
											15440
											15470
											15500
											15530
											15560
											15590
											15620
											15650
											15680
											15710
											15740
											15770
											1580

8/9

50										
Asp	Ala	Glu	Ile	Ala	Gly	Gly	Leu	Asp	Gln	
GAC	GCG	GAA	ATC	GCC	GGT	GGT	CTC	GAC	CAG	1056
80										
Glu	Gln	Pro	Leu	Asp	Met	Arg	Leu	Arg	Leu	
GAA	CAG	CCT	TTA	GAC	ATG	CGC	TTA	AGA	CTG	1152
110										
Tyr	Asn	Val	Ala	His	Thr	His	Gly	Gly	Ala	
TAC	AAC	GTC	GCA	CAC	ACC	CAC	GGC	GGT	GCA	1248
140										
Val	Ala	Gln	Ser	Lys	Ser	Glu	Thr	Tyr	Ser	
GTC	GCC	CAA	TCC	AAG	AGC	GAG	ACC	TAC	AGC	1344
180										
Gly	Gly	Gln	Lys	Ala	Ala	Gln	Arg	Trp	Lys	
GGC	GGA	CAA	AAA	GCC	GCA	CAA	CGC	TGG	AAA	1440
210										
Lys	Ala	Gln	Gly	Arg	Ser	Thr	Lys	Ser	Arg	
AAG	GCT	CAA	GGA	CGA	TCT	ACG	AAG	TCC	CGT	1536
240										
Ala	Glu	Val	Gly	Val	Ser	Arg	Ala	Thr	Val	
GCA	GAG	GTA	GGA	GTC	TCT	CGC	GCC	ACG	GTT	1632
AAG CAA TAT ACG GTT CCC CTG CCG TTA GGC										
T										
ACT	TCC	TCT	GTT	CTC	CTA	GAC	CTC	GCA	ACC	1824

Fig. 3f.

SUBSTITUTE SHEET

9/9

A B C D E F



A B C D E F G

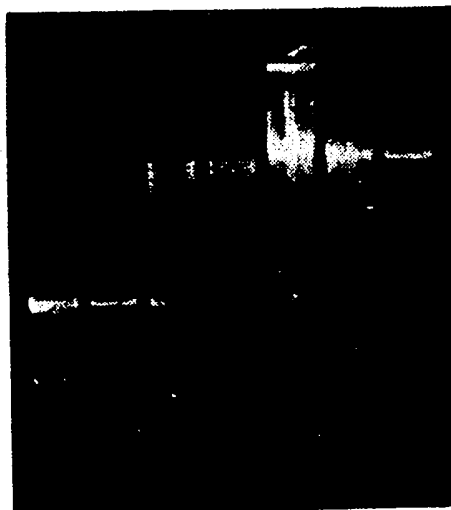


Fig.4.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 91/00064

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int. Cl. ⁵ C12N 15/74, 15/76, 15/77		
II. FIELDS SEARCHED		
Minimum Documentation Searched 7		
Classification System	Classification Symbols	
WPI/WPIL) Chem Abstr)	Keywords - MYCOBACTER:, VECTOR, PLASMID, SHUTTLE, CORYNEBACTER:	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8		
BIOTECHNOLOGY DATABASE KEYWORDS (as above) AU : C12N 15/74, 15/76, 15/77		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9		
Category*	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	WO,A1, 90/00594 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 25 January 1990 (25.01.90)	(1-6,8-10,21)
X	WO,A, 88/06626 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 7 September 1988 (07.09.88)	(1-6,8-10,21)
A	US,A, 4952500 (UNIVERSITY OF GEORGIA RESEARCH FOUNDATION INC) 28 August 1990 (28.08.90)	(1-21)
A	WO,A1, 90/10701 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 20 September 1990 (20.09.90)	(1-21)
<p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 7 June 1991 (07.06.91)		Date of Mailing of this International Search Report 7 June 1991
International Searching Authority Australian Patent Office		Signature of Authorized Officer <i>K. Ayers</i> K. AYERS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.